DEVELOPMENT OF A GENOME FINGERPRINT DATABASE TO IDENTIFY GENETICALLY ENGINEERED MICROBES

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ABSTRACT

The development of recombinant DNA technology and the current state of bioscience has for the first time made the laboratory creation of new biological weapons a real possibility. A critical part of any countermeasures is the ability to identify strains that are unlike previous strains that have been isolated, i.e. are "outside the box" of known genetic variability for the organism. A database of this type will help medical care providers plan an appropriate course of treatment and aid strategic decisions as to the possible origin of the new isolate. Such a database should be capable of including many types of data including source characterization and be capable of determining the phylogenetic relationship of any new isolate to the data contained in the database. We are using the Bionumerics software package and *Yersinia pestis* as our test organism. We examined 37 isolates from the United States (CONUS) and 20 isolates obtained from four different continents (OCONUS) that span a time period of 100 years. We examined our group of strains using a variety of techniques selective housekeeping gene sequencing and pulsed field gel electrophoresis (PFGE). We compared these methods with the established plague typing technique of ribotyping with ribosomal RNA gene probes. All of the CONUS strains belonged to a single ribotype. Chromosomal gene sequencing revealed that *Y. pestis* coding regions are highly stable and have not varied in any of the OCONUS strains. In contrast, PFGE was able to distinguish 46 strains (CONUS and OCONUS) that were derived from different parents. All CONUS strains were at least 70% similar to each other and could be divided into four groups that were greater than 90% similar. Furthermore, PFGE could distinguish one of two *Y. pestis* strains that were isogenic except for a single uncharacterized 14 kilobase pair insertion. Taken together, our results demonstrate that the *Y. pestis* genome is variable at the macromolecular level and that this instability is useful for determining the phylogenetic relationship between isolates and identification of potentially engineered BW strains.

INTRODUCTION

The likelihood that we will face the use of Genetically Engineered Microbes as biological weapons (BW) is significant (8). In order to counter this possibility one critical asset will be an information resource that can be used to identify strains that are different from previous isolates and have the capability of determining the geographic origin of the new isolate. The development of a genetic fingerprint database will support clinical investigation/treatment of patients as well as support strategic decisions pertaining to any possible military response. Several different laboratories associated with the Department of Defense, Department of Energy or Health and Human Services are conducting research to characterize the utility of various genetic techniques for the ability to discriminate between isolates. These techniques include variable number tandem repeats, DNA Sequencing of selected genes, pulsed-field gel electrophoresis (PFGE) and

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
00 JAN 2002	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Development Of A Genome Fingerprint Database To Identify Genetically Engineered Microbes	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Department of Bacterial Diseases, Division of Communicable Disease and Immunology, Walter Reed Army Institute of Research, Silver Spring, MD 20910**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
This article is from ADA409494 Proceedings of the 2001 ECBC Scientific Conference on Chemical and Biological Defense Research, 6-8 March , Marriott's Hunt Valley Inn, Hunt Valley, MD., The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**11**	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18

amplified fragment length polymorphism (2, 7, 9, 13). Accordingly, given the diversity of techniques being used, the analysis package and database software must be capable of incorporating many types of data for phylogenetic analysis. Together, we have chosen the Bionumerics software package by Applied Maths (Kortrijk, Belgium) as the framework for our fingerprint database.

We have chosen *Yersinia pestis* as our model BW threat organism. Little is known about *Y. pestis* genetic variability and the only current technique used to categorize isolates is ribotyping using 16S and 23S ribosomal DNA probes. Ribotyping is not satisfactory because there are only 20 described patterns following analysis of hundreds of isolates from different continents (5, 6). Previously, PFGE was used to determine the size of the *Y. pestis* genome (10). We have combined this technique with selected housekeeping gene sequencing (4) in order to begin to characterize the natural genomic variability of *Y. pestis*. Here, we report the results of those studies and the utility of these techniques for analysis of new plague isolates.

MATERIALS AND METHODS

Growth of bacteria and preparation of genomic DNA for analysis.

Bacteria were grown in Brain Heart Infusion (Difco Laboratories, Detroit, MI) broth supplemented with 2.5 mM $MgCl_2$ at 30^0C. When growth on solid medium was required the broth was supplemented with 1.5 percent agar. Whole-cell DNA was prepared from undiluted overnight cultures. Total genomic DNA was isolated using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN).

Genomic DNA for PFGE was prepared by diluting overnight cultures one to five with like medium. Plugs for PFGE were prepared by removing 1 ml of diluted overnight culture (OD_{600} ~ 0.2) and centrifuging at 5,000 x g for 5 minutes. The cells were washed once in ice cold PETT IV+E (1 M NaCl, 10 mM Tris, 20mM EDTA, pH 7.6) and suspended in 0.5 mls of like buffer. The cells were warmed at 37^0C for 1 minute and an equal volume of 1.4 percent PFGE sample preparation grade agarose (Biorad Laboratories, Richmond, CA) pre-warmed to 52^0C was added. The agarose-cell suspension was mixed rapidly and 100 µl aliquots were placed in the wells of disposable plug molds (Biorad) then allowed to solidify at 4^0C for 10 minutes. Groups of 10 similar samples were treated with 10 mls of lysis buffer (6mM Tris, 100mM EDTA, 1M NaCl, 1 mg/ml Lysozyme, 0.5% Sarkosyl, 0.2% Sodium Deoxycholate, pH 7.6) for 1 hour and 15 minutes at 37^0C. The samples were suspended in 10 mls of ESP buffer (500 mM EDTA, 1% Sarkosyl, 1 mg Proteinase K/ml, pH 9.2) and incubated at 55^OC for 6 hours to overnight. The ESP buffer was removed and the samples were washed twice with 10 mls of 10 mM Tris, 1 mM EDTA, 1 mM PMSF, pH 7.5 for one hour at room temperature. Finally, the samples were washed three times with storage buffer (10mM Tris, 50 mM EDTA, pH 8) for thirty minutes at room temperature before being stored at 4^0C.

Separation and analysis of genomic DNA by PFGE.

Genomic DNA prepared in agarose plugs was digested with *Spe*I restriction endonuclease overnight and fractionated by PFGE using a CHEF-DRII apparatus (Biorad). Electrophoresis was carried out in 0.5 X Tris-Borate-EDTA buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.3). Genomic fragments were separated on 1% agarose gels for 24-48 hours at 200 volts with a ramp from 10s to 30s. Gels were maintained at 7^OC for the length of the electrophoresis. Following electrophoresis, gels were stained in a solution of 1 µg/ml ethidium bromide for one hour followed by rinsing for 1 hour in water before visualization. PFGE patterns were analyzed for phylogenetic relationships with the Molecular Analyst software package (Biorad).

PCR amplification and sequencing of *Y. pestis* genes.

The oligonucleotide primers used to amplify the *Yersinia sp.* 16S rDNA, 16S-23S rDNA intergenic region, *mdh, asd, gyrA* and *galE* genes are listed in Table 1. The DNA sequences

TABLE 1. Primers used for amplification of *Y. pestis* gene sequences.

Gene	Primer Name and Sequence	Product Size[a] (bp)	GenBank Accession Number of *Yersinia* Sequences[b]	Source or Reference[c]
16S rDNA	8F AGTTTGATCATGGCTCAG 1448R CCATGGCGTGACGGGCAGTGTG	1407	AF282306, AF282307, AF282308	(14)
16S-23S Intergenic region (IGR)	UNI-1 CACACCGCCCGTCACACCAT UNI-2 TTAGCACGCCCTTCATCGCCTCTG	735	AF282218, AF282219, AF282220	BLAST of *Y. pestis* genome with *E. coli* 16S rDNA sequence (J01695)
mdh	MDH-1 TGGCCCGCAGGATGAGC MDH-2 TCTGCGATAGTAATGAGAATGTT	1452	AF282309, AF282310	BLAST of *Y. pestis* genome with *E. coli mdh* (Y00129)
galE	GALE-1 TGGCGTGCTATCTTTATT GALE-2 ATGAGGCGAGACCAATAC	1158	AF282311, AF282312, AF282313	BLAST of *Y. pestis* genome with *E. coli galE* (X06226)
gyrA	GYRA-51 ATGAGCGACCTTGCGAGAG GYRA-31 TGTTCCATCAGCCCTTCAATG	632	AF282314, AF282315	*E. coli gyrA* (X06744.1)
asd[d]	ASD-1 TCATATGCGGCTGTTTCC ASD-2 AGGCTACTGGCGTTTTCG	1894	AF282316, AF282317, AF282318	BLAST of *Y. pestis* genome with *E. coli asd* (V00262)

a- The PCR product size predicted following amplification of *Y. pestis* genomic DNA is indicated.

b- Accession numbers for *Y. pestis galE* sequences from the Angola, pestoides A and pestoides F strains (see Table 2) have not yet been deposited in GenBank.

c- GenBank accession numbers used to search the unfinished *Y. pestis* genome sequence are indicated in parenthesis.

d- Oligonucleotide primer pair asd13F (CCACGACACTATGCGACG) and asd18R (CCGCAACCCCCACTTACA) were used to amplify *Y. pseudotuberculosis* strains PB1/+ and 43 *asd* sequences. No PCR product was obtained with these bacteria when ASD1 and ASD2 primers were used in amplification reactions.

for *Y. pestis mdh, asd* and *galE* were obtained by searching the unfinished genome sequence at http://www.sanger.ac.uk/Projects/Y_pestis/blast_server.shtml with the *E. coli* protein sequences that correspond to the translated products for each of these genes. The sequence of the 16S-23S intergenic region (IGR) of the *Y. pestis* rDNA operon was obtained by searching the above genome database with the *E. coli rrnB* operon sequence. All BLAST (3) searches produced probability values of $6.3e^{-126}$ or greater. Amplification of the 16S rDNA sequences of *Y. pestis* was with previously described primers (14). Oligonucleotide primers for the amplification of *Y. pestis gyrA* were selected from the *E. coli* gene sequence. PCR primer pairs were selected from the *Y. pestis* sequences such that flanking DNA as well as the complete gene sequences would be

amplified. Amplification primer pairs and DNA sequencing primers were selected using PrimerSelect (Lasergene, Madison, Wisconsin) software. DNA sequencing reactions were performed using Perkin Elmer Applied Biosystems (ABI) dRhodamine Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase FS. Sequencing reactions were analyzed on an Applied Biosystems Incorporated (ABI) model 377XL DNA sequencer. DNA sequences were edited and assembled using Sequencher 3.0 software (Gene Codes Corporation, Ann Arbor, Michigan). Sequence alignment and phylogenetic tree building were with Megalign (Lasergene) using the Clustal method.

RESULTS

Genetic variation of *Y. pestis* strains at the nucleotide level.

All of the strains characterized in this study are listed in Table 2.

TABLE 2. List of isolates characterized in this study.

Strains[a]	Geographic origin or comments[b]	Biovar[c]	Year of isolation	Ribotype[d]	PFGE [e] group	*galE* Allele
Yersinia pestis						
KIM (pgm$^-$)	Iran	M	unknown	F	ND	1
A1122	CA, US	O	1939	B	ND	ND
NM59-BENZ	Bernalilo, NM, US	O	1959	B	2	ND
NM61-DURAN	Santa Fe, NM, US	O	1961	B	2	ND
NM66Jaramillo	Bernalillo, NM, US	O	1966	B	1	ND
AZ70-130-1	Apache, AZ, US	O	1970	B	1	ND
TX79-0209	Canyon, Texas, US	O	1979	B	2	ND
NM81-3387-684	NM, US	O	1981	B	2	ND
NM82-0395	Rio Arriba, NM, US	O	1982	B	2	ND
NM83-0854	San Miguel, NM,US	O	1983	B	ND	ND
NM85-4298-585	Bernalillo, NM,US	O	1985	B	ND	ND
NM87-2987-614	Cibola, NM, US	O	1987	B	2	ND
NM87-1298	Rio Arriba, NM, US	O	1987	B	2	ND
NM87-2007	McKinley, NM, US	O	1987	B	2	ND
NM95-1065	Santa Fe, NM, US	O	1995	B	ND	ND
NM95-1100-276	Santa Fe, NM, US	O	1995	B	3	ND
CO96-3188CAT	Larimer, CO, US	O	1996	B	4	ND
NM96-3002-658	Bernalillo, NM, US	O	1996	B	2	ND
CO96-3188	Larimer, CO, US	O	1996	B	4	ND
NM96-2968	Bernalillo, NM, US	O	1996	B	3	ND
CO96-1214	El Paso, CO, US	O	1996	B	3	ND
NM96-3404	Bernalillo, NM, US	O	1996	B	2	ND
NM96-2970	Bernalillo, NM, US	O	1996	B	2	ND
NM97-2129-373	Santa Fe, NM, US	O	1997	B	2	ND
NM97-2129-374	Santa Fe, NM, US	O	1997	B	2	ND
NM97-2072-344	Santa Fe, NM, US	O	1997	B	3	ND

NM97-2064-338	Santa Fe, NM, US	O	1997	B	2	ND
NM97-2070-345	Santa Fe, NM, US	O	1997	B	3	ND
NM98-0152	Albuquerque, NM	O	1998	B	ND	ND
NM98-2993	Santa Fe, NM, US	O	1998	B	2	ND
NM98-2993Org	Santa Fe, NM, US	O	1998	B	2	ND
NM98-2446 Small	Santa Fe, NM, US	O	1998	B	2	ND
NM98-2446 Large	Santa Fe, NM, US	O	1998	B	2	ND
NM98-2252	Santa Fe, NM, US	O	1998	B	4	ND
NM98-0510-86	Santa Fe, NM, US	O	1998	B	2	ND
NM98-0511-87	Santa Fe, NM, US	O	1998	B	2	ND
NM98-1714	Santa Fe, NM, US	O	1998	B	2	ND
CO99-1133	Larimer, CO, US	O	1999	B	2	ND
Angola	Angola	ND	<1985	ND	ND	2
PEXU 2	Brazil	O	1966	B	ND	1
Harbin 35	China	M	1940	F	ND	1
195/P	India	O	1898	B	ND	1
17721	India	O	1994	ND	ND	1
516	Nepal	ND	1969	F	ND	1
F361/66	South Africa	ND	1966	ND	ND	1
16-34	Vietnam	ND	1970	G	ND	1
ZE942122	Zimbabwe	ND	1994	B	ND	1
219	Vietnam	O	?	ND	ND	1
A16	Belgian Congo	O	?	ND	ND	1
CO92	Colorado	O	1992	ND	ND	1
La Paz	Bolivia	O	?	ND	ND	1
PMB9	Burma	O	1984	ND	ND	1
Stavropol	Russia	O	?	ND	ND	1
Antigua	Belgian Congo	A	1954	ND	ND	1
PKR108	Kurdistan	M	?	ND	ND	1
Yersinia pestoides						
15-91	Russia	?	1960	ND	ND	1
Pestoides A	Russia	?	?	ND	ND	4
Pestoides F	Russia	?	?	ND	ND	3
Yersinia pseudotiberculosis						
PB1/+	Type 1 strain	NA[f]	?	NA	NA	6
43	Type III	NA	?	NA	NA	5

a. For all US isolates (except A1122), the first two capital letters represent states. The number following that represents the year the organism was isolated. The number after the first dash represents the ID number of the patient or animal. Two dashes indicate that the isolates were from fleas with the number after the second dash indicating the flea ID number.

b. NM=New Mexico; CO=Colorado; TX=Texas.

c. The Biotyping information was provided by Dr. P. Worsham, USAMRIID, Frederick, MD. O=Orientalis; M=Medievailis.

d. Ribotyping is according to a method described previously (5).

e. ND= not determined.

f. NA= not applicable.

We examined the *mdh, galE, gyrA, asd*, 16S rDNA and IGR gene sequence variation for a diverse group of *Y. pestis* strains. In the first group of experiments, we determined the DNA sequence of

all of these genes in nine OCONUS *Y. pestis* islolates and two different strains of *Yersinia pseudotuberculosis*. The plague strains were PEXU2, Harbin, 195/P, 17721, 516, F361/66, 16-34, ZE94-2112 and KIM5 listed in Table 2 and were obtained from Dr. May Chu at the Centers for Disease Control, Fort Collins, Colorado. The total number of basepairs analyzed was approximately 65,000 and we did not identify a single nucleotide change within this group of *Y. pestis* strains. Single nucleotide polymorphisms (SNP) were identified when the *Y. pestis* and *Y. pesudotuberculosis asd*, *galE* and *gyrA* sequences were compared. In contrast, the 16S rDNA and IGR sequences between these two species were identical. Next, we examined a larger group of strains obtained from Dr. Patricia Worsham in the Bacteriology Division, U.S. Army Research Institute of Infectious Disease (USAMRIID). The second group of *Y. pestis* strains examined for SNP was Angola, 219, A16, CO92, La Paz, PMB9, Stavropol, Antigua, 15-91, pestoides Type A, pestoides Type F and PKR108 listed in Table 2. None of these strains encoded any SNP within *mdh* compared to the first group of *Y. pestis* we examined. However, *galE* encoded nucleotide changes at four positions. Combinations of these changes resulted in six different alleles of *galE* within these *Y. pestis* and *Y. pseudotuberculosis* strains as indicated in Table 2. The most predominat DNA sequence of *galE* was designated as allele number 1. The only *galE* sequence variation within *Y. pestis*-like organisms was seen in strains Angola, pestoides Type A and pestoides Type F. Figure 1 shows a phylogenetic tree of the *galE* sequences we derived. All of the "atypical" *Y. pestis* strains were found to fall between the majority group of typical strains and *Y. pseudotuberculosis*.

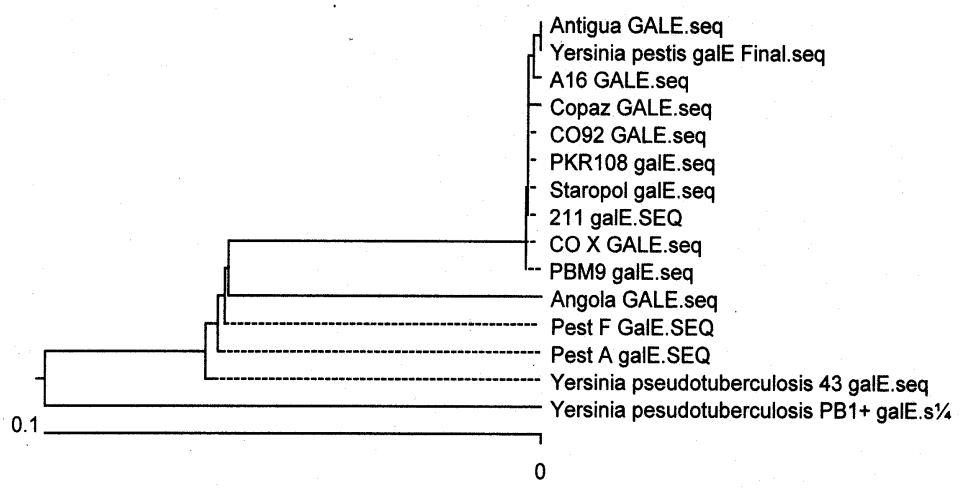

Figure 1. Dendogram of *Yersinia galE* DNA sequences. Allele numbers were assigned from the majority group at the top to the most distant member shown at the bottom of the tree. Sequence alignment was with Megalign (DNAStar) and the Clustal algorithm.

PFGE analysis of CONUS and OCONUS *Y. pestis* strains.

We analyzed most of the *Y. pestis* strains listed in Table 2 by PFGE as described in the Materials and Methods above. All of the OCONUS plague strains we examined displayed a unique PFGE profile as shown in Figure 2 below.

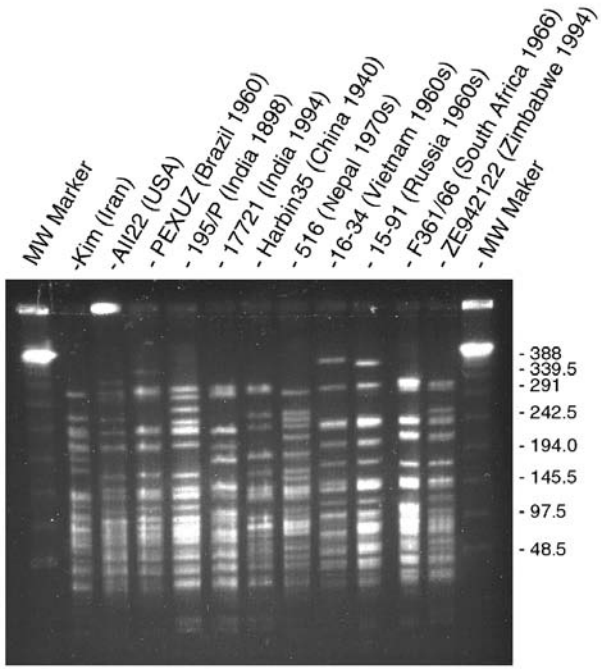

Figure 2. PFGE of representative *Y. pestis* strains of diverse origin. Genomic DNA in agarose plugs was digested overnight with *Spe*I and separation was as described in the Materials and Methods. Electrophoresis was for 24 hours. Molecular weight (MW) marker was applied to the first and last lane with the sizes of the individual fragments shown at the right of the figure. Sizes of the MW marker fragments are in kb.

The amount of heterogeneity seen when we examined our group of CONUS strains using similar conditions to those used in Figure 2 was much lower. Many of the strains displayed more than 90% similarity. Most of the CONUS strains fit within four groups as shown in Figure 3 below. The only strains within the CONUS group that could not be differentiated were ones that either originated from the same parent or were isolated from the same region in the same year, i.e. were clones of each other. These results suggested that the genome is highly variable in the natural environment but that the variation seen in laboratory sub-culture is very low. Evidence of the independent evolution of the genotypes of *Y. pestis* can be seen in Figure 3. For example, the PFGE pattern of NM98-2252 is very different from NM98-1714 yet both isolates originated from Santa Fe in 1998. This apparent independent evolution of PFGE patterns may be due to the natural endemic foci of plague in the US. Specifically, *Y. pestis* is endemic in Prairie Dog colonies and these groups may not intermingle with other groups. Humans become exposed to plague by infected fleas from these colonies and therefore the human isolates would be expected to carry the genotype of the organism from the colony that caused the infection. The number of possible genotypes may be limited. Examination of the data shown in Figure 3 reveals that some geographically diverse strains encoded similar PFGE profiles. For example, comparison of strain TX79-0209 with NM98-1714 revealed that these isolates greater than 90% similar but were obtained 19 years apart and from two different states. We found that regional strains did not group together, i.e. strains obtained from a particular city were interspersed with strains isolated from other cities (Figure 3).

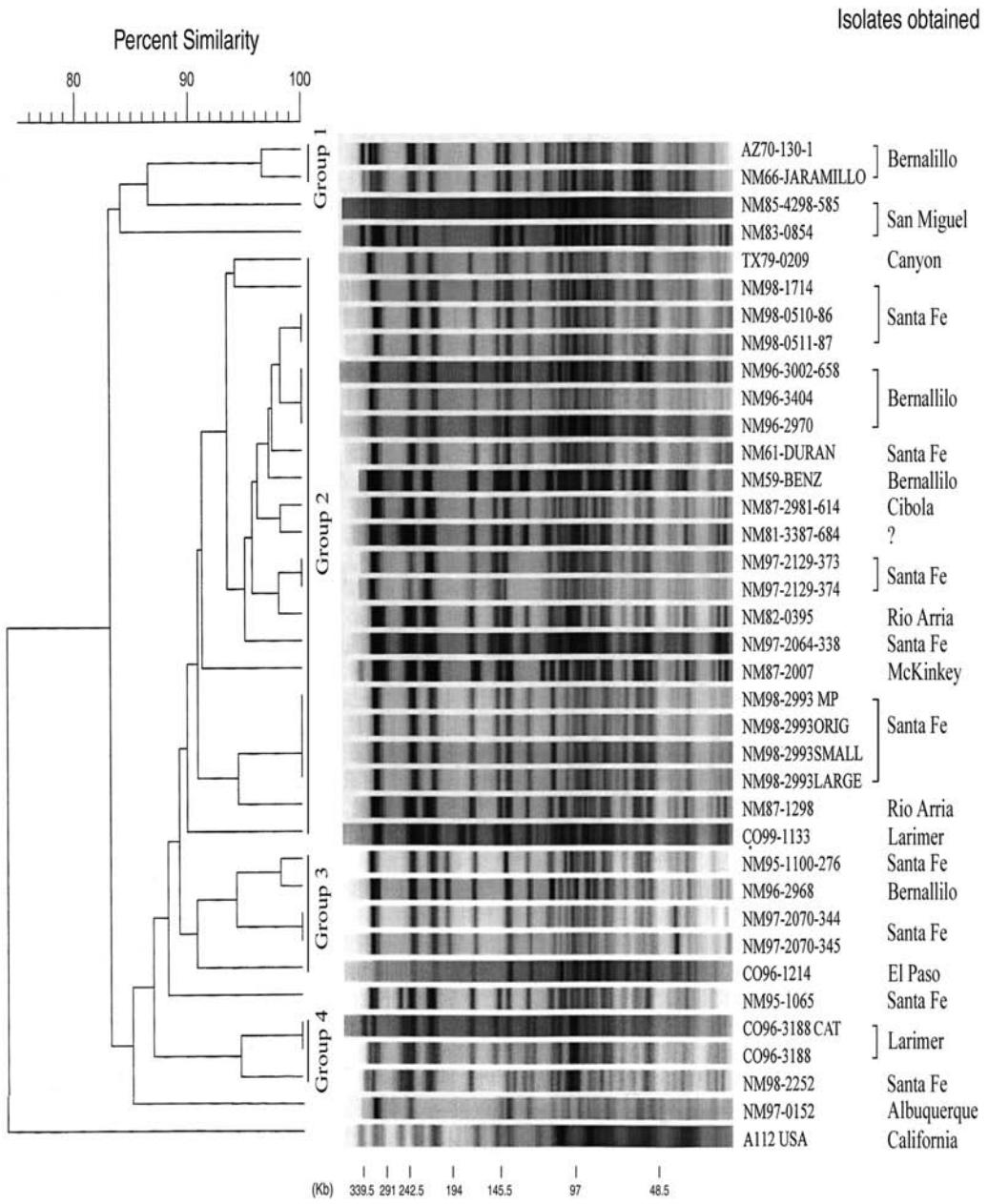

Figure 3. Dendrogram from digitized PFGE patterns for the 37 domestic *Y. pestis* isolates digested with *Spe*I was constructed by cluster analysis using the Dice coefficient and the Molecular Analyst Software Version 1.6 (BIO-RAD Laboratories, Richmond CA). Percentage of similarity is shown above the dendrogram. The patterns are ordered from least similar to most similar (left to right). The position tolerance was 1.4%. The ID numbers of the US isolates are as listed in Table 2.

Detection of genetically modified strains of *Y. pestis*.

Since one of the principle goals of this project was to determine if we could identify potentially modified BW agents we examined two *Y. pestis* mutants generated in our laboratory. These mutants were generated by random mutagenesis of *Y. pestis* KIM5 with Tn10-*lacZ* using bacteriophage λ as a delivery vehicle (15). TN10-*lacZ* is approximately 14 kb in size and was

inserted randomly in the *Y. pestis* KIM5 genome. Approximately 10,000 mutants were screened for pH-regulated β-galactosidase expression at 37^{O}C and two mutants were identified that displayed this phenotype. The mutants were designated numbers 13 and 15 and have not been further characterized. We compared the PFGE pattern of mutants 13 and 15 with the pattern produced by the isogenic parental *Y. pestis* strain KIM5 and identified a single restriction fragment that was not present in mutant 15 as shown in Figure 4 below. The loss of the approximately 230 kb fragment in mutant 15 was accompanied by a gain of an approximately 240 kb fragment. These same strains have been analyzed by the VNTR technique in the laboratory of Dr. Paul Keim at the University of Northern Arizona (2) and were found to be identical by this technique (Paul Keim personal communication). Accordingly, PFGE was able to distinguish a genetically modified *Y. pestis* strain from the parental strain in contrast to more modern PCR-based techniques.

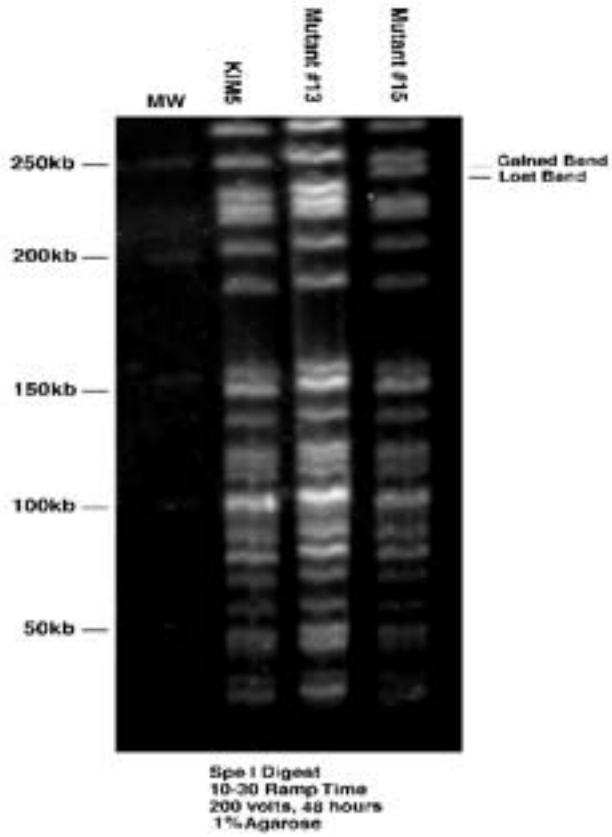

Figure 4. PFGE analysis of TN10-*lacZ* insertions in *Y. pestis* KIM5. The contents of each lane is labeled above gel. Electrophoresis conditions are shown below the image and the size of molecular weight (MW) markers is shown to the left. The DNA fragment that differentiated mutant 15 from the parent strain is indicated to the right of the image.

CONCLUSIONS

We have examined the genetic variability of *Y. pestis* within a diverse as well as homogeneous group of strains by PFGE and by DNA sequencing of selected housekeeping genes. Our analysis indicates that the organism is highly variable at the whole genome level since PFGE profiles of geographically close isolates could be distinguished in most cases. One area of concern we identified was the similarity between a few isolates obtained from geographically dissimilar

regions. This may indicate that the *Y. pestis* genome is not infinitely plastic at the whole genome level. The reason for similar PFGE profiles obtained from geographically dissimilar strains remains unclear however it must be considered that the strains in our phylogenetic comparison shown in Figure 3 were all obtained from the continental US. Unfortunately the PFGE patterns obtained for our OCONUS strains were analyzed using different conditions than the CONUS strains. Accordingly, a more comprehensive comparison of PFGE patterns has not been possible to date but is planned for the near future.

Our finding that *Y. pestis* is very homogeneous at the nucleotide level must be considered in light of the whole genome fingerprint variability noted above. SNP has been used routinely with other enteropathogens to determine clonal and phylogenetic relationships (12). However in the case of the analysis of the genetic relationship of *Y. pestis* strains by this technique all of the "typical" isolates would be considered one clone. Furthermore, the "atypical" strains (Angola and the two pestoides types) are intermediate between *Y. pestis* and *Y. pseudotuberculosis* as might be predicted by earlier studies (1). Our demonstration that "atypical" *Y. pestis* strains are more related to *Y. pseudotuberculosis* by housekeeping gene sequencing is in agreement with other PCR-based techniques (2 and DR. Patricia Worsham personal communication).

Our demonstration that PFGE can differentiate at least some genetically modified BW agents is encouraging. However, the choice of restriction enzyme and the conditions of the fragment separation directly influence the ability of this technique to identify small changes in otherwise isogenic strains. We must therefore increase our experience with PFGE conditions as well as obtain a comprehensive dataset of profiles against which we can compare future isolates.

To date we have used the Molecular Analyst software marketed by Bio-Rad Laboratories for our genetic relationship analysis. However, we are migrating our data to the Applied Maths Bionumerics suite for a more comprehensive ability to incorporate different types of data into our ability to distinguish strains that are significantly different from previous isolates, i.e. have emerged as new clones or are geographically "out of place". This same software package is in use by CDC as part of their Pulsenet program to monitor *Escherichia coli* O:157 isolates for newly emerging strains (11). This package is suitable for a Biodefense database because it has the ability to accommodate many types of data including gel patterns of any kind, biochemical characteristics, antigenic profile and DNA sequences. Furthermore, investigators at remote sites can deposit their data as well as perform analysis using data contained in the central database. We are currently working with Dr. Ted Hadfield at AFIP and Dr. May Chu at CDC in Fort Collins, CO to begin to build our database capabilities for many of the BW bacterial pathogens.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. May Chu, Dr. Patricia Worsham and Dr. Ted Hadfield for providing strains and DNA for these analyses. We would also like to thank SPC Sydney Lee and Ying Liu for database work and technical assistance, respectively.

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